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Simplified Fluctuation Test to Distinguish Liquid Holding Recovery from Cell Multiplication in Ultraviolet-Irradiated *Escherichia coli*

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Improved plating techniques and a fluctuation test have been used to distinguish between liquid holding recovery of ultraviolet-irradiated cells and cell multiplication in *Escherichia coli*. Among tested strains, only *recA* mutants showed true recovery.

An increase in the survival of irradiated cells, due to the partial recovery of induced lesions, is observed during buffer incubation (10). This phenomenon, called "liquid holding recovery," is dependent on the *uvr* gene products and is best expressed in *recA* mutants (4). During liquid holding, the enzyme polymerase I seems to play a major role in increase in viability of the cells (12, 13).

Turbidimetric measurements and direct counting of cells with a Petroff-Hauser chamber have indicated the absence of cell division (11). Recently, multiplication of irradiated and nonirradiated buffer-incubated bacterial cells has been described; this is probably due to reutilization of proteins from damaged cells or reduction of the internal pool, or both (2, 3, 7). Actually, in experiments in which liquid holding recovery was measured, the majority of the population was formed by nonviable cells. As a consequence, it was necessary to use improved methods to distinguish between recuperation of viability (true liquid holding recovery) and cell multiplication; some of these methods are presented and discussed in this paper.

The strains used were *Escherichia coli* K12S, *E. coli* K12A16 (*uvrA16*), *E. coli* B/r, obtained from Institut Curie, Paris, France, and *E. coli* AB2463 (*recA13*), provided by P. Howard-Flanders (6). *E. coli* K12S, *E. coli* K12A16, and *E. coli* B/r were grown in M9 medium (1), and *E. coli* AB2463 was grown in supplemented M9 medium, at 37°C with aeration (up to 5×10^8 cells per ml). These cells were recovered by filtration (Sartorius membrane filter; pore size, 0.45 μm), washed in 67 mM phosphate buffer (PB), pH 7, and suspended in the same buffer. UV irradiation and dosimetry were done as described previously (2), with the dose rate adjusted to 2.5 W m^{-2} (for doses above 100 J m^{-2}) and 0.02 W m^{-2} (for doses below 100 J m^{-2}). Although preirradiation treatments, such as incubation in buffer at 37°C for 2 h (4) or 2 h of incubation at room temperature followed by overnight incubation at 4°C (12), decreased the photosensitivity of the cells, they did not alter our final results. All of the manipulations and incubations after irradiation were performed under yellow light. Platings were done in either nutrient agar or eosin methylene blue-glucose agar (8).

For incubation in solidified buffer, samples from irradiated preparations were either mixed with 5 ml of PB containing agar (0.75%) and added to petri dishes or directly spread on plates with PB-agar (1.5%) for further incubation at 37°C. Periodically the number of viable cells was determined by adding 6 ml of eosin methylene blue-glucose containing 0.75% agar (samples mixed with PB), by respreading and further plating, or by replica plating (samples spread directly). Similar experiments were performed with either washed agar or washed agarose. For incubation on nitrocellulose membranes, 1-ml samples of irradiated cultures were filtered in a Sartorius membrane filter (diameter, 25 mm; pore size, 0.45 μm). These membranes were incubated in a water vapor-saturated chamber at 37°C, soaked with PB by capillarity, and periodically suspended in PB and plated in eosin methylene blue-glucose solidified medium or directly placed on the same medium. Colonies were counted after 24 h of incubation at 37°C.

For evaluation of increase in viability, using a fluctuation test based on Poisson distribution, irradiated cultures were properly diluted to obtain a mean of less than one viable cell per 50 μl immediately after irradiation. Samples, 50 μl, were distributed in five groups of 60 small tubes (30 by 5 mm) each and incubated at 37°C in the dark. At 0, 2, 4, 6, and 24 h, 200 μl of nutrient broth was added to each group of test tubes. The tubes were then reincubated for 24 h at 37°C. Each group was analyzed to determine the

fraction of the total number of tubes in which cell multiplication did not occur (absence of turbidity). The average number of viable cells per tube was deduced from the Poisson distribution (9).

Figure 1A presents the relative increase of population as a function of incubation time of *E. coli* K12S cells irradiated with 120 J m^{-2} (survival fraction, 4×10^{-4}) under four different experimental conditions: (i) incubation in PB (liquid phase); (ii) trapping in agar phosphate (0.75%); (iii) trapping in agarose phosphate (0.6%); and (iv) incubation on a Sartorius membrane filter. Only the cells which were free in the buffer showed an increase in the number of colony-forming units. These data indicate that the relative increment of increase in cell populations incubated in liquid buffer should be associated with multiplication of the remaining viable cells and not with repair.

Cell multiplication has been observed, however, during incubation on nitrocellulose membranes or in gelled buffer, as measured by a respreading technique (Fig. 1B). As the experimental conditions used were not identical to incubation in liquid medium, we used a fluctuation test based on the Poisson distribution (9).

Table 1 shows the fraction of tubes in which *E. coli* K12S cells did not grow [$P(0,\mu)$] and the average number of cells in each tube (μ). These values remained constant during the 24 h of incubation, whereas those for the control population increased 80-fold. If this increment was due to repair, the probability of null events [$P(0,\mu)$] would be almost zero after 24 h of incubation, since the value of μ, based on the increase in cell number, is approximately 16 and $P(0,\mu) = e^{-\mu}$.

TABLE 1. *Cell multiplication in E. coli K12S during liquid holding*[a]

Time (h)	Probability of null events [$P(0,\mu)$]	Avg no. of cells per tube (μ)	Relative viability during LH
0	0.81	0.21	1
2	0.75	0.29	1.2
4	0.80	0.22	1.3
6	0.82	0.20	2
24	0.74	0.30	80

[a] *E. coli* K12S was irradiated with UV (dose, 120 J m^{-2}; survival fraction, 4×10^{-4}) and incubated in buffer, and the relative increase in viability was expressed by the relation between the number of colonies formed after liquid holding (LH) and the initial number. For determination of $P(0,\mu)$, see text.

The fluctuation test was used to study the increase in viability of *E. coli* B/r, *E. coli* AB2463, and *E. coli* K12A16 incubated in buffer after UV irradiation, as described previously (2, 4, 5). Table 2 shows the relative increase in the population during liquid holding and the average

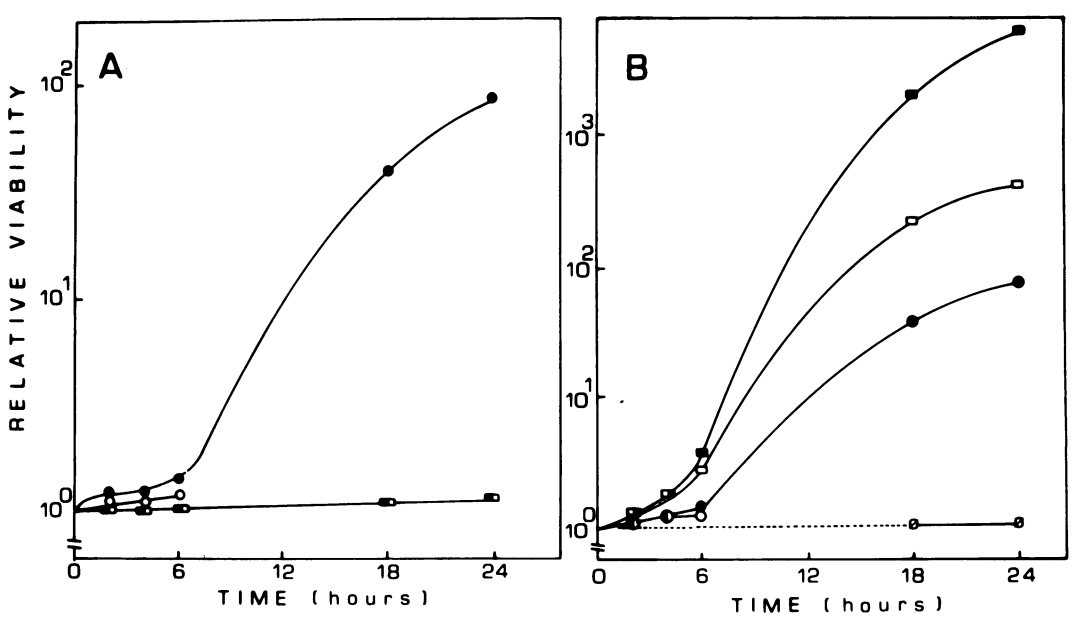

FIG. 1. *Relative viability of UV-irradiated E. coli K12S cells during liquid holding. (A) Increase in viability as a function of initial number of cells when:* (●) *incubated in liquid phase,* (■) *incubated in agar phosphate,* (□) *incubated in agarose phosphate,* (○) *incubated on a nitrocellulose membrane. (B) Same as (A) after respreading.* (□) *Replica plating from agar or agarose phosphate.*

TABLE 2. *Relationship between liquid holding (LH) recovery and cell multiplication in E. coli B/R, E. coli K12A16, and E. coli AB2463*[a]

Time (h)	*E. coli* AB2463		*E. coli* B/r		*E. coli* K12A16	
	μ (cells per tube)	Relative viability during LH	μ (cells per tube)	Relative viability during LH	μ (cells per tube)	Relative viability during LH
0	7×10^{-3}	1	0.27	1	0.12	1
2	0.36	15	0.24	0.8	0.18	1.8
4	2.10	86	0.20	0.7	0.18	2
6	2.99	220	0.21	0.9	0.16	3.5
24	9.33	860	0.24	41	0.18	17

[a] *E. coli* AB2463 was irradiated with 9 J m^{-2} (survival fraction, 3.4×10^{-5}); *E. coli* B/r, with 130 J m^{-2} (survival fraction, 10^{-4}); and *E. coli* K12A16, with 20 J m^{-2} (survival fraction, 4.0×10^{-4}). For details, see Table 1, footnote *a*.

number of cells per tube (μ), using the fluctuation test, in *E. coli* B/r irradiated with 130 J m^{-2} (survival fraction, 10^{-4}), *E. coli* K12A16 irradiated with 20 J m^{-2} (survival fraction, 4×10^{-4}), and *E. coli* AB2463 irradiated with 9 J m^{-2} (survival fraction, 3.4×10^{-5}).

As can be seen, the true liquid holding recovery was found only in *E. coli* AB2463. In the other strains the relative increase in the population during liquid holding was promoted by cell multiplication. This kind of multiplication should be observed when prototrophic cells, irradiated or not, are incubated in buffer for a long time.

Based on these results, we can conclude that the distinction between cell multiplication and liquid holding recovery may be an important step in studying the increase in viability of UV-irradiated prototrophic cells.

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